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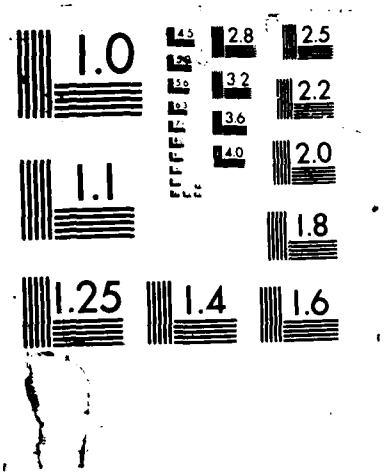
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The goal of this research is to understand the barrier properties of lipid bilayers, and particularly the role of transient defects in providing pathways for ion flux. Our approach is to compare proton and potassium flux, using membrane perturbants to probe the barrier. We have found that perturbants such as n-alkanols and diols cause increments in cation flux, which can be used to predict anesthetic effects. The increments are caused by increased numbers of hydrated defects, and we have used the gramicidin channel to test properties of transmembrane strands of hydrogen bonded water which would model such defects. Results are described in the report.

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## INTRODUCTION

The lipid bilayer moiety of biological membranes represents a barrier to ion flux which permits most membranes to maintain ion concentration gradients essential for function, such as the electrochemical proton gradients of coupling membranes and the sodium-potassium ion gradients of axons. However, the bilayer is not a perfect barrier, and there are measureable fluxes of protons and metal ions like sodium and potassium. The nature of the barrier and the mechanism of conductance have intrinsic interest. Both involve physical properties of the bilayer, particularly in regard to transient defects that permit ionic flux to occur. In past work, we have reported that protons have intrinsic permeabilities five to six orders of magnitude greater than that of other cations. Protons appear to have a unique pathway for flux across the bilayer, and we suggested that the conductance mechanism may involve Grotthus conductance along hydrogen bonded water associated with the membrane. In the progress to be reported here, we extended this work to gramicidin A, which forms ion conducting channels containing single strands of hydrogen bonded water molecules. We found that these channels had very high proton permeabilities relative to other ions. This leads to the intriguing possibility that biological membranes may have similar proton flux mechanisms related to their function, an example being the Fo subunit of coupling membranes. The primary goal of our research is to expand our knowledge of ionic flux mechanisms, with emphasis on proton flux, and to link such conductance pathways to membrane function.

## PROGRESS REPORT

About half of the reporting period (August 1, 1986 - March 1, 1987) was spent at the Australian National University, Canberra, while on sabbatical leave at the Department of Applied Mathematics. Research supported by the Office of Naval Research was continued at UC Davis by Mr. John Mais, our Staff Research Associate, and 2 months of work was carried out at ANU on ion conductance measurements made in media with different dielectric constants. Progress was made in the following areas: 1. Gramicidin as a model "proton wire." 2. Effects of homologous series of alcohols on proton flux in liposomes, and the relationship to their anesthetic properties. 3. Application of the above findings to proton permeability and proton pumping in synaptic vesicles.

### 1. Gramicidin as a model proton wire.

Gramicidin A is a pentadecapeptide which forms channels consisting of two pi-helices in head-to-head contact (1,2). The helices have a 0.2 nm channel along their long axes, sufficient to contain a single strand of hydrogen bonded water molecules (3 ). It has long been suspected that this channel would permit proton flux through a hydrogen bond exchange mechanism (4 ) but very little work has been carried out. Our approach was to test this hypothesis by direct comparison of proton and potassium flux through the channel, using the results to determine relative permeabilities at physiological pH ranges. We could then compare the properties of the channel to proton/potassium flux in lipid bilayers, which we have postulated also occurs through a wire-like conductance.

To summarize our results, we found that the intrinsic proton/potassium permeability ratio was in the range of 10 E4. This was less than the six orders of magnitude difference we see in lipid bilayers, but impressive nonetheless. If the channel does contain hydrogen bonded water strands, it would be expected that channel conductance to protons would be reduced by exchanging water for deuterium oxide, since it is known that deuterium oxide ice is significantly less conductive than water ice. When this measurement was made, we found that proton flux in deuterium oxide was reduced by a factor of two, while potassium flux was unaffected. If we are able to show that such an isotope effect is a general property of proton wires, it will be a useful tool to explore the possible existence of wire-like conductance in biological membranes.

2. Effects of homologous alcohols on proton/potassium permeabilities of lipid bilayers.

If the intrinsic permeability of a lipid bilayer depends on transient defects occurring in the hydrocarbon chains through thermal motions, it would be expected that the number of such defects would be increased by certain perturbant molecules. In past work supported by ONR (5 ) we showed that this is indeed the case: molecules such as chloroform, halothane, diethyl ether and n-butanol all increased the relative permeability of lipid bilayers to protons and potassium. Furthermore, we could relate this observation to a theory of general andsthesia proposed in 1980 by Bangham and Mason (6 ) which suggests that the primary anesthetic effect is on proton permeability of synaptic vesicles, thereby reducing their ability to maintain gradients of catecholamine neurotransmitters.

In order to better understand the effects of such perturbants, we have investigated a series of alcohols up to four carbons, including diols and triols. There were several surprises. First, we found that glycerol had essentially no effect on permeability, despite earlier reports to the contrary. This could be explained by the partition coefficient of glycerol - even at 7 M concentrations in the aqueous phase, insufficient amounts get into the bilayer phase to produce significant perturbations of the low dielectric barrier. We also found that 1,2 butanediol and 1,4 butanediol had somewaht different effects on permeability. That is, the 1,2 diol approximately doubled permeability at 0.5 M, while the 1,4 diol required nearly 2 M concentrations to produce the same effect. This result was unexpected, since the 1,4 diol has a higher partition coefficient and would be expected to reach 4-fold greater membrane concentration. This observation led to a prediction that the 1,2 diol would have anesthetic activity, but not the 1,4 diol. We tested this in Medaka fish, with a positive result. This is the first time an anesthetic effect has been predicted from proton permeation rates, and represents evidence favoring the Bangham pump-leak hypothesis.

3. Effects of general anesthetics on synaptic vesicles

The results summarized above were sufficiently exciting that we decided to extend the effort to a more biological system. Synaptic vesicles are an obvious choice, and we have established methods for preparing synaptic vesicles from the bovine caudate nucleus. With this system, we will be able to critically test the pump leak hypothesis by monitoring the effects of anesthetics on ATPase activity, pH gradients, proton permeability, and catecholamine uptake associated with synaptic vesicle membranes.

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1. Deamer, D.W. (1987) Proton Permeation of Lipid Bilayers. J. Bioenerg. Biomemb. 19:457-479.
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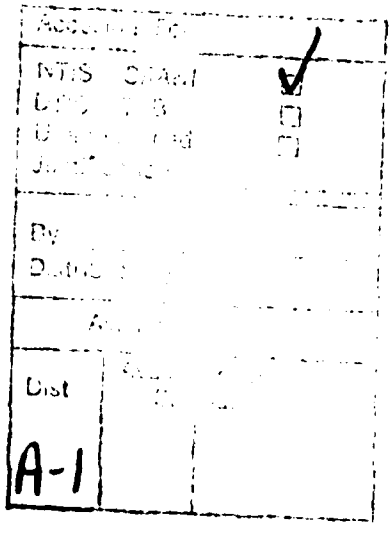
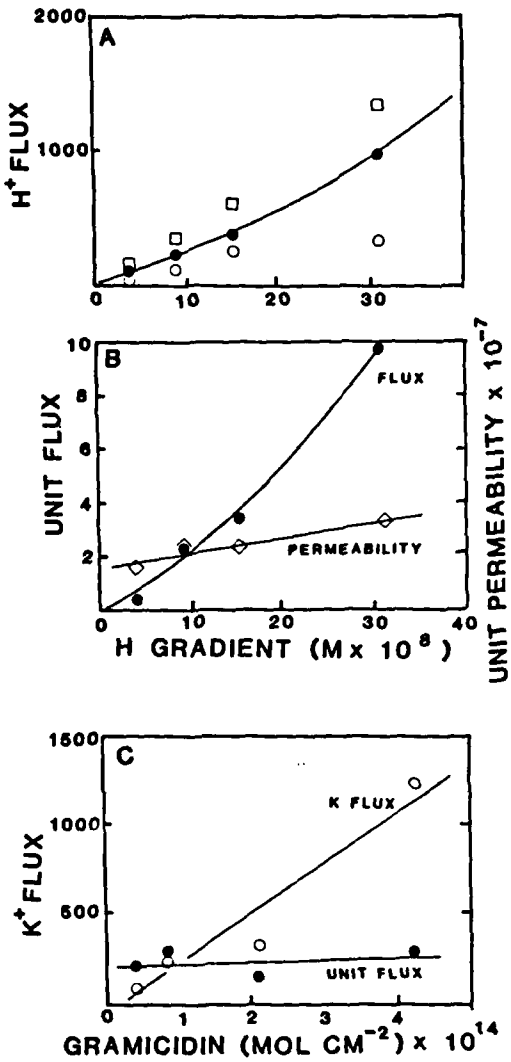
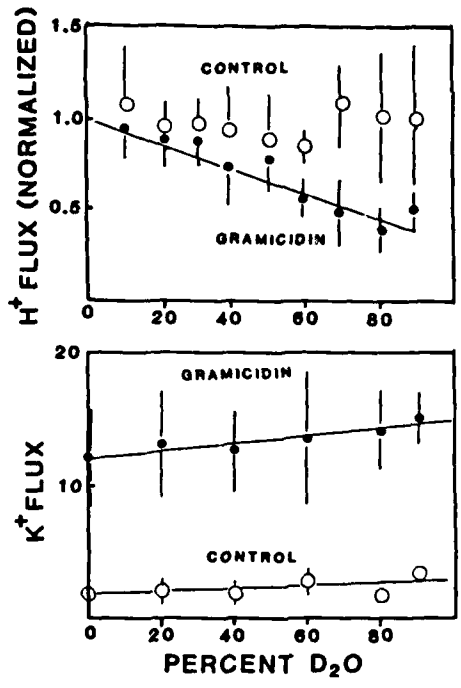
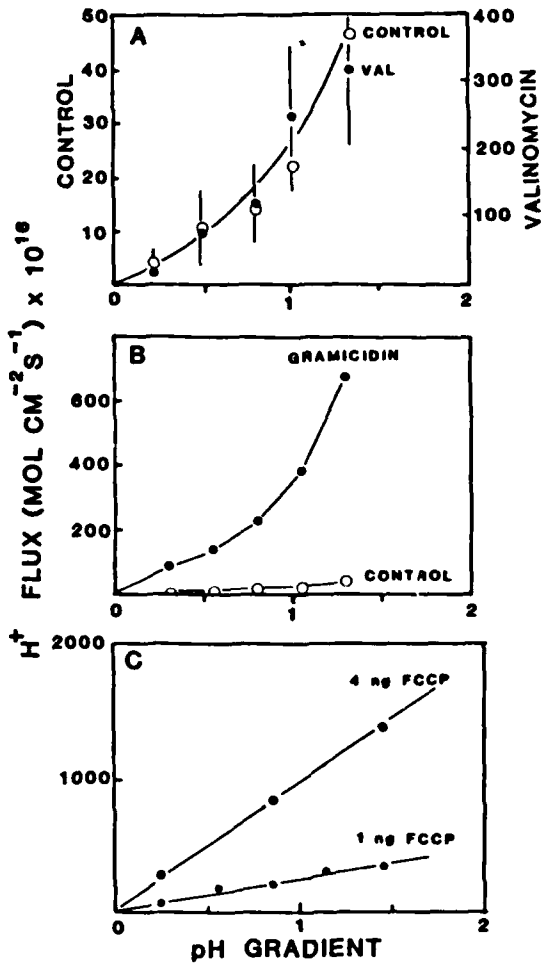





Relative permeability of gramicidin channels to protons and potassium ions. Proton flux (mol $cm^{-2} s^{-1} \times 10^{16}$) was measured as described in the text and Fig. 1, and potassium flux (mol $cm^{-2} s^{-1} \times 10^{14}$) was measured by a potassium-sensitive glass electrode, with choline replacing potassium in the external medium. Valinomycin (0.5 μg/mg lipid) was present during proton flux measurements to prevent proton diffusion potentials from interfering. Gramicidin concentration was 100 ng/mg phospholipid ($1.05 \times 10^{-14}$ mols channel $cm^{-2}$ bilayer) for proton flux measurements (egg PC:POPA liposomes, 9:1, 1 mg total lipid). Gramicidin was varied from 0 to 100 ng in potassium flux measurements (5 mg total lipid), and its concentration in the figures is given as mols channel $cm^{-2} \times 10^{14}$. To measure proton flux, gramicidin concentration was kept constant and pH gradients were varied from 0.3 to 1.3 pH units across liposome membranes (A). The open circles show control values, and open squares show proton flux with gramicidin present. The closed circles and line give the difference, which was taken to represent proton flux through gramicidin channels. Potassium flux was measured with a gradient of 0.4 M potassium ion, and gramicidin was varied as shown in (C). From the flux increments caused by gramicidin additions, unit flux could be calculated as ions per channel per second. This ranged from 1 to 10 protons per second as the pH gradient increased from 0.3 to 1.3 pH units (B) and was approximately 230 potassium ions per channel per second (C) The unit permeability coefficients were then calculated as unit flux/concentration, giving values of $3 \times 10^7$ for protons and 585 for potassium ions, with a $H^+/K^+$ permeability ratio of $5 \times 10^4$.




Effect of $D_2O$ on proton and potassium flux. Proton flux was monitored by the pyranine method described in the text and Fig. 1, and potassium flux was measured with a glass electrode as described in Fig. 3. Because of approximately 2-fold variation between liposome preparations, the proton flux was normalized, with values in 100% $H_2O$ taken as 1.0. Means of four runs + S.D. are shown, and lines are drawn by standard linear regression analysis. Potassium flux is given as mol cm $^{-2} \times 10^{14}$. The results show a clear trend to lower proton flux through the gramicidin channel as $D_2O$ increased, but no effect in the absence of gramicidin. Potassium flux was not affected by $D_2O$.




Relation between proton flux and pH gradient in liposomes. Proton flux was measured as described in Fig. 1. (A) The means of four separate experiments, for controls and valinomycin additions (0.5 μg/mg PL). Standard deviations are given for the valinomycin data. The line is a theoretical curve from model C plotted from the equation $J = J_0(\sinh[\beta\delta])$, where $\beta = 1/kT$ and $\delta = 2.3kT\Delta pH$. (See Nagle, this volume.) (B) An experiment in which gramicidin (0.1 μg/mg PL) was present, and comparison of the plot of flux vs pH gradient against a control experiment using the same liposome preparation. (C) Internal buffer was increased to 50 mM in order to measure higher flux rates, and valinomycin (0.5 μg/mg PL) and two concentrations of FCCP (1 and 4 ng/mg PL) were present. The plots of flux against pH gradient appear linear.

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